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Device and method for homogenising small-volume chemical reaction mixtures

The present invention relates to the field of chemical reactions requiring temperation and/or incubation of the reaction mixture and in particular chemical reactions in liquid media. The present invention discloses a new device and method for efficient and homogenous mixing, temperation and/or incubation of reaction mixtures.

Background of the invention

Many important industrial processes as well as procedures applied in laboratories of various kinds are dependent on chemical reactions. Commonly the time consumed for completing a process or procedure is determined by the time it takes for some specific chemical reaction or reactions to reach equilibrium. This is often referred to as the kinetic properties of a chemical reaction or simply reaction kinetics. A host of variables influence the reaction kinetics in each case, for instance the concentrations of reactants, temperature, presence of catalytic agents etc.

Typically, increased temperature makes chemical reactions faster by speeding up key mechanisms like bringing molecules or molecule domains in contact with each other. Therefore it is common to heat the reaction vessels, for example bringing them in contact with an open flame, hot gas, hot liquid, hot sand or a solid material. This procedure is often referred to as incubation.

One typical problem involved with incubations of fluid reaction mixtures is thermal heterogeneity, because the parts of the reaction mixture being in close contact with the walls of the reaction vessel will become heated before the more central parts of the reaction mixture. In many cases there is a risk of part of the reaction mixture becoming overheated before other parts even reach the desired temperature. This give raise to temperature gradients in the reaction mixture. Hot subsets of the reaction mixture has normally lower density than cold subsets which tend to generate temperature gradients or discrete layers of more or less isothermal bodies of liquid, so called thermoclines. Thus warm, less dense portions of the reaction mixture tend to find a position above cold, denser portions. Molecular motion and currents in the reaction mixture will eventually homogenise the reaction mixture with respect to temperature, a process referred to as temperation of the reaction mixture. The time it takes to temperate a reaction mixture may contribute substantially to the time required for the complete reaction.

However, time-consumption is not the sole problem involved with temperation of chemical reaction mixtures. In certain incubation procedures such as the repetitive temperations involved in so called thermocycling processes, e.g. for performing polymerase chain reactions, that is PCR-reactions, long temperation periods also leads to unwanted side-reactions, sometimes causing severe quality problems.

In the ongoing strive to miniaturise chemical reaction volumes, as evident e.g. in the field of high throughput screening, several other problems are encountered. In a small reaction vessel, such as a well on a microtitre plate, both the mixing and temperation of sample and reagents may become severely restricted. When two or more miscible fluids are mixed, we normally assume that they first form a homogenous mixture, which then reacts. This is however rarely the case.

Conventional microtitre plates and cuvettes are often manufactured from polystyrene, a hydrophilic polymer. Without dwelling on the exact behaviour of the liquid at the vessel boundaries, it can be concluded that stagnant areas will form and insufficient mixing easily occur in a small reaction vessel, such as a well on a microtitre plate. The properties of the reactants and sample fluids also influence their interaction with each other and with the vessel boundaries. Partial segregation, the formation of layers, aggregation and so on are only a few examples of irregularities that can be encountered in a reaction vessel.

There are reasons for distinguishing between two different processes causing problems with heterogeneous temperature distribution in a reaction mixture. The process caused by the lower fluidity close to the walls of a reaction vessel is a problem increasing when reaction scale decreases. In contrast, the problem involved with central parts of the liquid body being colder than close to walls when heating a reaction vessel, increases when reaction scale increases. This is the reason why thermocycling devices for processes in which proper temperation is required (e.g. processes like PCR) has a very narrow dynamic range with respect to the reaction scale. Typically, in PCR-reactions these problems are most severe when reaction volumes are less than 5 μL and larger than 50 μL.

Another problem, seemingly unrelated to the mixing and temperation issues, is that of evaporation. In order to avoid evaporation, there exists a tendency to make the reaction vessels and in particular the wells on microtitre plates deeper and more narrow. Naturally, this further enhances the previously mentioned problems of insufficient mixing and temperation.

So far, temperature heterogeneity has been discussed in terms of properties in a single reaction vessel. Especially when discussing miniaturisation of assays yet another dimension of temperation heterogeneity need to be considered; that of variation between reaction vessels. In assays with comparative purposes with or without quantitative analysis like screening for novel drug candidates, mutations in nucleic acids, single nucleotide polymorphism and so forth it is important to consider the reproducibility, commonly referred to as well-to-well uniformity.

Since the processes leading to poor thermal uniformity are difficult quantitatively to predict, the often only available solution to the problem is to focus on means to enhance the homogenization processes. To do this, various strategies are applied.

One is to use reaction vessels with specific flat or oblong configurations in order to minimise the distance between the central and peripheral parts of the bulk of the reaction mixture. An example of this is to perform the incubation in thin capillaries as described by Wittwer, C.T. *et al.* (The LightCycler™: A Microvolume Multisample Fluorimeter with Rapid Temperature Control, *BioTechniques* 22:176-181, January 1997). One disadvantage with this approach is that the glass capillaries, loosely attached to their plastic holding portions, require extensive manual handling. The choice of glass capillaries makes possible both rapid temperation of the reaction mixture and detection of fluorescence after amplification. However, a glass capillary tends to maximise the surface-to-fluid contact, with the consequences this has on mixing and temperation.

Other ways to solve the problem with mixing and temperature heterogeneity is to introduce perturbation by agitating or shaking the reaction vessel. Specific instruments designed for this purpose are for example various kinds of flask shakers and so called Vortex machines. A problem often encountered with this approach is that the perturbation periodicity may cause currents or standing waves and therefore incomplete homogeneity. Another approach for homogenisation is the use of ultrasonic waves in a standard procedure called sonication. The latter procedure is, unfortunately, often difficult to combine with a number of standard incubation methods.

Prior art

WO 98/49340 (PCT/AU98/00277) discloses a temperature cycling device and method where a reaction mixture and a sample is loaded into loading wells on a disposable rotor, which rotor is then placed into a centrifugal thermal cycling device and spun, so that the

reaction mixture and sample are moved by centrifugal force to a reaction well at the periphery of the rotor. The device comprises heating means, for example infrared lights, convection heating elements or microwave sources. Interestingly, also provisions for cooling the rotor are included in the specification. According to one embodiment, the rotor speed is increased, resulting in air being drawn into the device and rapidly cooling the contents of the reaction chambers at the periphery of the rotor. In addition to ambient air, a coolant gas can be used. Refrigerated air is given as an example of coolant gases. Importantly, the disclosure does not address the problems of mixing and homogenous temperation. For example, it does not specify the direction of heating, nor does it contemplate simultaneous heating and cooling.

It was the purpose of the present invention to solve the problem with time consuming and insufficient mixing and temperation steps in procedures where incubation of small-volume chemical reaction mixtures are involved by finding means for effective mixing and homogenisation. Importantly, said means should be compatible with means for unhindered analysis of the reaction and preferably also applicable in existing and future technologies in the fields of chemical and biotechnological reactions, screening and analysis, such as high throughput screening, amplification reactions etc.

Summary of the invention

This problem is solved by the invention according to the attached claims 1 through 4, defining a novel device. The invention further discloses a method for performing chemical reactions according to the attached claims 5 through 9.

Short description of the figures

The present invention is described below with reference to the attached drawings in which

Fig. 1 shows cross sections of three main types of rotors, suitable for use in a device according to the present invention;

Fig. 2 shows a schematic cross section of a device according to the invention; and

Fig. 3 shows in cross section a rotor comprising means for simultaneous heating and cooling.

Description

In the following description of the invention, certain definitions will be used. They are to be interpreted as outlined below:

The direction of the gravitational field: Described with vectors, the direction of the gravitation field is the same as the resulting vector when a vector representing the centrifugal force, that is in right angle to the axis of the centrifuge rotor directed from the centre of the rotor, is added to the vector representing the gravitation of earth. Consequently, downwards is in the present text defined as the same direction as the gravitation field as defined by the summed vectors representing centrifugal force and gravitation.

Reaction mixture: Any fluid reaction mixture, preferably a liquid reaction mixture, in which the reaction kinetics are influenced by temperature and where a faster, more efficient and homogenous temperation is desired. Examples of reactions, suitable for the present device and method are chemical / biochemical reactions within the field of high throughput screening and biochemical reactions involving repeated temperation, e.g. cyclic temperature changes, including a polymerase chain reaction (PCR), a ligase chain reaction (LCR), a "gapped-LCR-reaction", a nucleic acid sequence-based amplification (NASBA), a self-sustained replication (3SR), a transcription mediated amplification (TMA), a stand displacement amplification (SDA), a target amplification, a signal amplification, or a combination of any of the above.

Typically a polymerase chain reaction involves the following steps:

1) Preparation of the reaction mixtures, i.e. preparation of the samples to be tested;
2) Amplification, i.e. the exponential replication of the DNA molecules; and
3) Detection of specific sequences for example by electrophoresis or hybridisation.

Step 2) involves repeated temperature changes to take the reaction mixture through the steps of annealing and extension of the nucleotide strands. Inefficient temperation, that is diffuse temperatures in the reaction mixture leads to unspecific amplification products. The necessity of a fast and homogenous temperation of the reaction mixture is central for the quality and reliability of the reaction.

Reaction vessel: Any vessel capable of containing a reaction mixture within the temperature range necessary for performing the reaction. Examples of reaction vessels suitable for use according to the invention include, but are not limited to, the following: test tubes, so called micro tubes, Eppendorf-tubes, a single well or a multitude of wells in a microtitre plate, such as a microtitre plate of the 96-hole format, and various formats with a high density arrays of wells, such as the 192-hole format, the 384-hole format or the like. The reaction vessel according

to the present invention can be a conventional, commercially obtainable reaction vessel as listed above, or a reaction vessel specially adapted for use in the inventive device, for example including an optical element at its distal end, a special colour or surface for absorbing heat at its distal end or having opaque, isolating or reflective surfaces on its side walls.

The reaction mixture is situated in a reaction vessel, e.g. a vessel having at least one opening and one closed end, substantially in the opposite direction of the open end. Said vessel is loaded with at least one reactant, which by definition constitute the reaction mixture which mixture is to be homogeneously incubated. Reactants are dispensed through the open end which after dispensing may be sealed, for example using a lid. Dispensing of reagents may be performed using manual or automated pipetting devices or, in a preferred embodiment of the invention, using predispensed reagent capillaries (PCT/SE91/00343) or reagent cartridges (PCT/SE97/01562).

The closed end is defined as the distal end pointing in the same direction as the current gravitation field. The open end or the end closed with a lid or reagent cartridge, is called the proximal end. Thus, when no centrifugation occur, the distal end of a reaction vessel is often directed downwards in accordance of the gravitation field of the earth. In congruence with this statement, upwards in the present text is defined as the direction in opposition to the gravitation field that affects the reaction vessel with content in a specific situation. The proximal end is consequently directed upwards or in a direction opposite to the centrifugal force.

A device according to the present invention comprises means (1', 1'' and 1''') for holding at least one reaction vessel (3', 3'' and 3''') with contents; means for subjecting the said at least one reaction vessel and its contents to a centrifugal force; means (2', 2'' and 2''') for heating the part of the reaction vessel directed outwards in relation to the centrifugal force; and means for cooling the proximal end of the reaction vessel (not shown).

Preferably said means for holding at least one reaction vessel is chosen among the following: a drum rotor (schematically illustrated as 1' in Fig. 1), a swing-bucket rotor (1'') and a fixed angle rotor (1'''). Further, the chosen rotor is preferably modified to allow unhindered thermal contact between the distal or "lower" end of the reaction vessels and the heating source. Likewise, the rotor is suitably modified to allow efficient cooling of the proximal end.

The heating source (2', 2'' and 2''') can be any heating source, capable of heating the distal ends of the reaction vessels, e.g. a radiating source, such as a heating element with electric resistance wires, a IR-source, a microwave element and the like. Preferably, the heating source is adapted to the shape of the rotor, as schematically indicated in Fig. 1 (see e.g. 2'''). The heating source may also be situated inside the rotor, at the distal or lower ends of the reaction vessels (not shown). According to one embodiment of the invention, a telecentric lens is positioned between the heating source and the reaction vessel or reaction vessels.

The cooling source or means for cooling can be chosen among convection cooling and a circulating cooling medium, e.g. a refrigerated gas, such as air and preferably nitrogen.

The reaction vessels can be any one of the following: a set of individual tubes, e.g. Eppendorf-tubes (illustrated as 3' in Fig. 1), individual wells on a microtitre plate (3'') or individual test tubes (3''').

In Fig. 2 the device is shown from above, illustrating a rotor (1) surrounded by a heating element (2) and two examples of reaction vessels, a microtitre plate (4) and a test tube (5) placed so that the length axis of each vessel (or individual well in case of the microtitre plate) is orientated approximately in the direction of the centrifugal force vector. The means for cooling (10) or refrigeration is indicated schematically.

In Fig. 3 is shown a rotor having an inlet (6) and an outlet (7) for a cooling medium circulating past the proximal part of the reaction vessels. Suitable cooling mediums include commonly used cooling fluids, such as pure gases or gas mixtures, for example liquid nitrogen or cold air. The heating element (9) is only schematically indicated.

According to one embodiment of the invention, said device is capable of subjecting the contents of the reaction vessel or vessels to a centrifugal force in the interval of about 50 x g to about 1500 x g. The temperation achieved by the present inventive device or method is naturally a function of the reaction volume, its constitution, the temperature and the centrifugal force.

Further, a device according to the invention comprises means for cooling the part of the reaction vessel directed inwards in relation to the centrifugal force. In this case, the rotor is modified to allow unhindered thermal contact between the proximal or "upper" end of the reaction vessels and the cooling medium.

The means for heating comprise at least one source of heat, for instance a radiation source emitting radiation within a wavelength range generating heat, an electric element, hot gas or hot liquid, is positioned within a centrifuge in such way that the heat when emitted, reach the distal end or ends, of one or several reaction vessels when these reaction vessels, being appropriately situated in the rotor of the centrifuge, are centrifuged. When the invention is intended for cyclic heating and cooling, this source of heat can be switched on and off without terminating the centrifugation. The effect of the heat source should be high enough to bring the compete amount of reaction mixture to be contained in the reaction vessel or reaction vessels, to the temperature being appropriate for the desired chemical reaction. The heating means can preferably comprise a temperature sensor or sensors or a thermostat for monitoring and controlling the heating.

The means for cooling can comprise means for leading a cooling medium, e.g. a gas, liquid or ambient air into close proximity of the proximal or "upper" end of the reaction vessels. A suitable cooling medium is liquid nitrogen or refrigerated nitrogen gas.

When the invention is used, reaction vessels including the complete reaction mixture or a subset of this, are placed in the rotor of the centrifuge with the closed end directed downwards or otherwise according to standard practice for centrifuging the reaction vessels in question. The centrifuge is then started, that is, the engine which brings the rotor to spin is switched on. When the rotor has accelerated give to the chosen gravitation force, the rotation is kept at constant speed. The heating source is now switched on leading to increased temperature predominantly at the apices of the reaction vessels. The heat will be transferred through the material of the walls of the reaction vessels, to the most distal part of the bulk of the reaction mixture. Increased molecular motion due to increased temperature will expand, that is, decrease the density of this heated part of the reaction mixture. Due to pressure caused by the gravitation field acting on more dense subsets of the reaction mixture, the parts with lower density will be forced to move upwards, immediately replaced by reaction mixture with higher density. This dense part will then be heated by means of the same process of heat transfer from the heating source. The density of this part of the reaction mixture will decrease and move upwards and become replaced by cooler reaction mixture. The means for cooling, acting simultaneously on the upper or proximal part of the reaction vessel will co-operate to ensure thorough mixing and homogenisation. This chain

of events will carry on, eventually leading to a thoroughly mixed, homogenous reaction mixture and a homogeneous temperature distribution in the bulk of the reaction mixture.

The simultaneous cooling from the opposite direction of the gravitation field efficiently increases the degree of density homogenisation, that is, shortens the time for temperation and homogenisation of the reaction mixture.

A method according to the invention comprises the following steps:

i) at least one reactant is measured into a reaction vessel;

ii) said reaction vessel with contents is placed in a device capable of subjecting it simultaneously to centrifugation and heating; and

iii) said reaction vessel is subjected to centrifugation

iv) the distal end of the reaction vessel is subjected to heating simultaneously with cooling of the proximal end.

According to a preferred embodiment of the invention at least one reactant is added using a capillary or similar device, which only releases its content upon centrifugation.

In general, the present invention also discloses a new method for performing chemical reactions in fluid media contained in reaction vessels, characterized in that a device according to any one of claims 1 – 4 is used.

In particular, the present invention discloses a novel and efficient method for performing reactions demanding a high degree of homogenisation and involving repeated temperation e.g. analyses and synthesis involving thermocycling. One example of such analyses is the polymerase chain reaction (PCR) technique. Another important application is in the field of high throughput screening. This and other methods are outlined previously in the description.

Although the invention has been described with regard to its preferred embodiments, which constitute the best mode presently known to the inventors, it should be understood that various changes and modifications as would be obvious to one having the ordinary skill in this art may be made without departing from the scope of the invention which is set forth in the claims appended hereto.

Claims

1. Device for use in the performing of chemical reactions in fluid media contained in reaction vessels, **characterized** in that said device comprises

- means for holding at least one reaction vessel with contents;
- means for subjecting the said at least one reaction vessel and its contents to a centrifugal force;
- means for heating the part of the reaction vessel directed outwards in relation to the centrifugal force during ongoing centrifugation; and
- means for cooling the part of the reaction vessel directed inwards in relation to the centrifugal force during ongoing centrifugation;.

2. A device according to claim 2, **characterized** in that said means for holding at least one reaction vessel is chosen among the following: a drum rotor, a swing-bucket rotor and a fixed angle rotor.

3. A device according to any one of the claims above, **characterized** in that the reaction vessel is chosen among the following: a micro tube, an Eppendorf-tube or a well in a microtitre plate.

4. A device according to any one of the claims above, **characterized** in that a telecentric lens is positioned between the heating source and the reaction vessel or reaction vessels.

5. Method for performing chemical reactions in fluid media contained in reaction vessels, **characterized** in that said method comprises the following steps:

i) at least one reactant is measured into a reaction vessel;

ii) said reaction vessel with contents is placed in a device capable of subjecting it simultaneously to centrifugation, heating and cooling of opposite ends of the reaction vessel; and

iii) said reaction vessel is subjected to simultaneous centrifugation, heating and cooling of opposite ends of the reaction vessel.

6. A method according to claim 5, **characterized** in that at least one reactant is added using a capillary or similar device, which only releases its content upon centrifugation.

7. Method for performing chemical reactions in fluid media contained in reaction vessels, **characterized** in that a device according to any one of claims 1 – 4 is used.

8. A method for performing biochemical reactions involving thermocycling analysis, **characterized** in that a device according to any one of claims 1 – 4 is used.

9. A method for performing biochemical reactions involving thermocycling, **characterized** in that said method comprises the following steps:

i) at least one reactant is measured into a reaction vessel;

ii) said reaction vessel with contents is placed in a device capable of subjecting it simultaneously to centrifugation, heating and cooling; and

iii) said reaction vessel is subjected to simultaneous centrifugation, heating of the distal end, and cooling of the proximal end of the reaction vessel;

wherein step iii) is repeated as many times as necessary, optionally with an additional cooling step between the heating steps.

Abstract

A device for aiding in the performing of chemical reactions in fluid media contained in small-volume reaction vessels, characterized in that said device comprises means for holding at least one reaction vessel with contents; means for subjecting the said at least one reaction vessel and its contents to a centrifugal force; and means for heating the part of the reaction vessel directed outwards in relation to the centrifugal force and cooling the part of the reaction vessel directed inwards in relation to the centrifugal force during ongoing centrifugation. The invention further relates to a method for performing chemical reactions and in particular a method for performing biochemical reactions involving thermocycling, for example PCR-reactions.

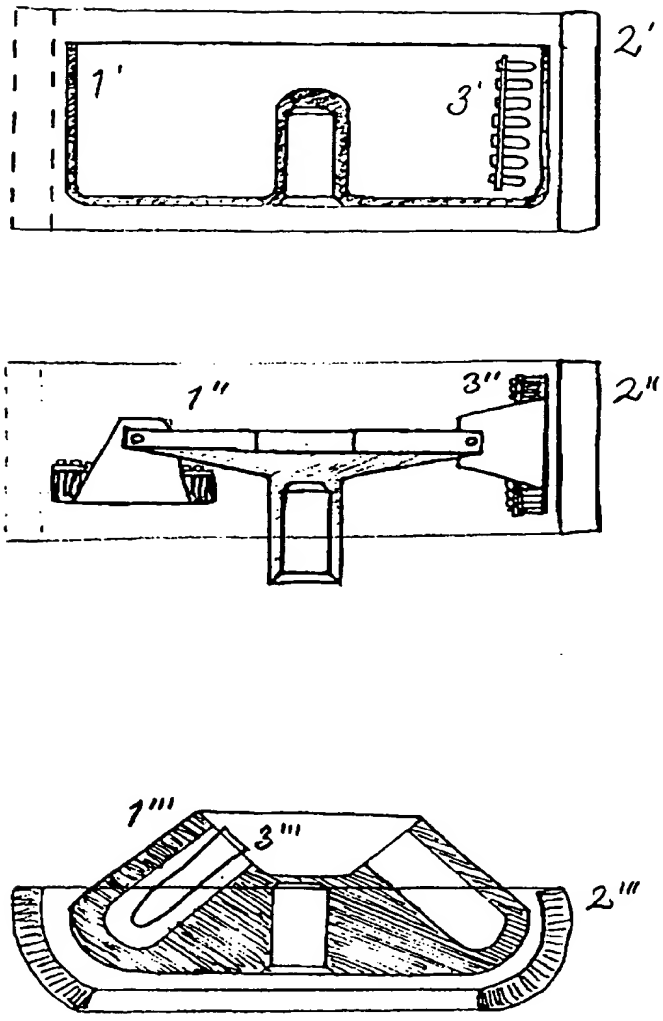

Fig. 1

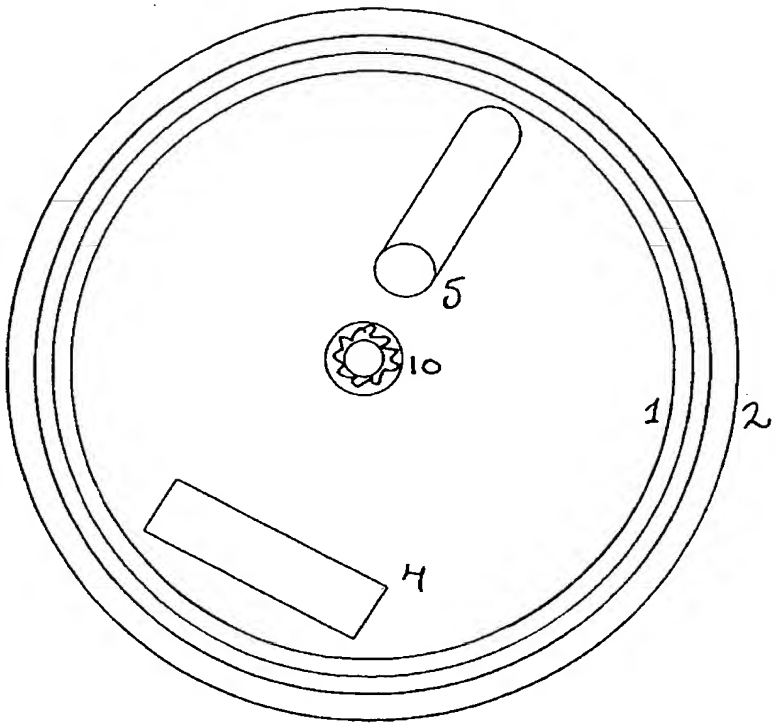

Fig. 2

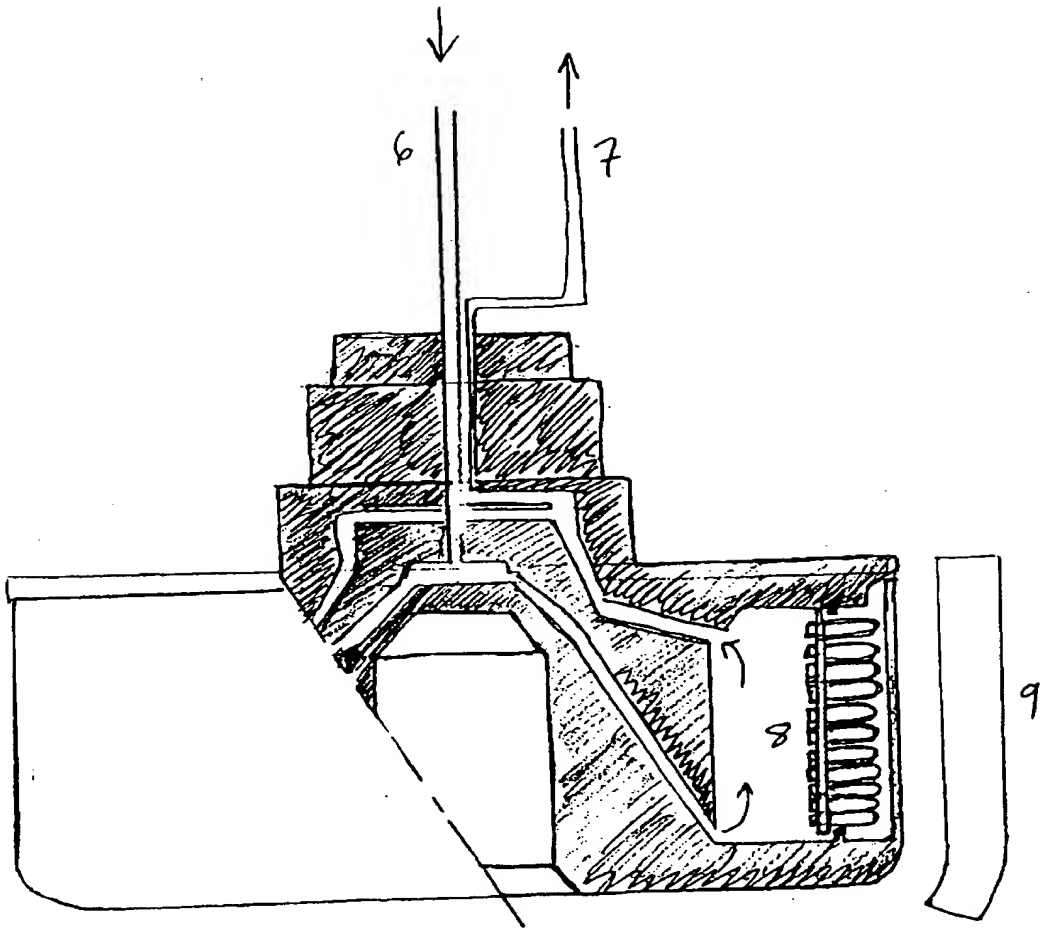

Fig. 3